Timing and coordination of DNA replication in *Escherichia coli*

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"What is true for *E.coli* is true for the elephant"

Jacques Monod

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Acknowledgements

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Solveig Fossum

List of papers

I Fossum S, Søreide S and Skarstad K (2003) Lack of SeqA focus formation, specific DNA binding and proper protein multimerization in the *Escherichia coli* sequestration mutant *seqA2*. *Mol Microbiol,* **47,** 619-632.

II Odsbu I, Klungsøyr HK, Fossum S, and Skarstad K (2005) Specific N-terminal interactions of the *Escherichia coli* SeqA protein are required to form multimers that restrain negative supercoils and form foci. *Genes Cells,* **10,** 1039-1049.

III Fossum S, Crooke E and Skarstad K (2007) Organization of sister origins and replisomes during multifork DNA replication in *Escherichia coli*. Submitted.

IV Fossum S, De Pascale G, Weigel C, Messer W, Donadio S and Skarstad K (2007) A robust screen for novel antibiotics: Specific knockout of the initiator of bacterial DNA replication. Submitted.

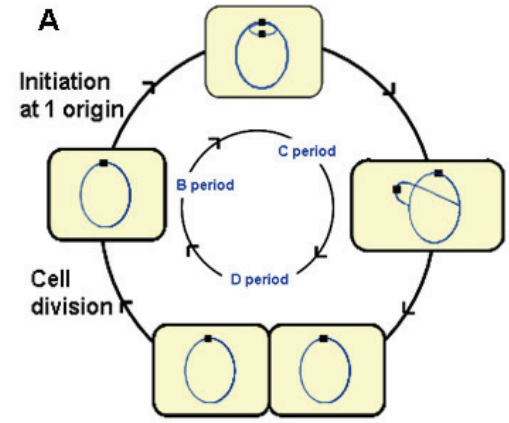
Introduction

The bacterium *Escherichia coli* (*E.coli*) a gram-negative, non-spore forming, facultative anaerobic, rod-shaped bacteria from the family Enterobacteriaceae. During the 20th century the *E.coli* bacteria has become one of the best understood forms of life and constitutes a unique potential for understanding the physiology of other prokaryotic and eukaryotic cells.

The E.coli *cell cycle*

The *E.coli* cell cycle consists of cell growth, duplication of the genome and cell division into two identical daughter cells. Slowly growing bacteria have a cell cycle that is quite similar to that of eukaryotic cells containing G1, S and G2 phases (Boye *et al.*, 1996). In bacteria these periods are called B, C and D periods (Fig 1A) (Cooper and Helmstetter, 1968). The B period is the time from birth of the cell until initiation of DNA replication. The replication time, which includes initiation, elongation and termination, is called the C period and the time from termination of replication to the end of cell division is called the D period. The C and D periods are relatively constant for a given strain at growth rates shorter than 60 minutes; C is typically about 40 minutes and D is about 20 minutes. In cells growing with a doubling time longer than 60 minutes, the duration of the C and D periods increases significantly (Skarstad *et al.*, 1983;Skarstad *et al.*, 1985).

E.coli is in rich medium capable of very rapid growth with doubling times around 20 minutes. Under such conditions the replication pattern is quite different because the time it takes to replicate the chromosome is not reduced correspondingly. Therefore, when the time it takes to replicate and segregate the chromosome exceeds one generation, a new round of replication is initiated before the previous round is completed. This results in multifork DNA replication (Cooper *et al.*, 1968). A schematic illustration of a cell that initiates at four origins early in the “grandmother” generation is shown (Fig 1B).


A
Initiation
at 1 origin
C period
B period
D period
Cell
division

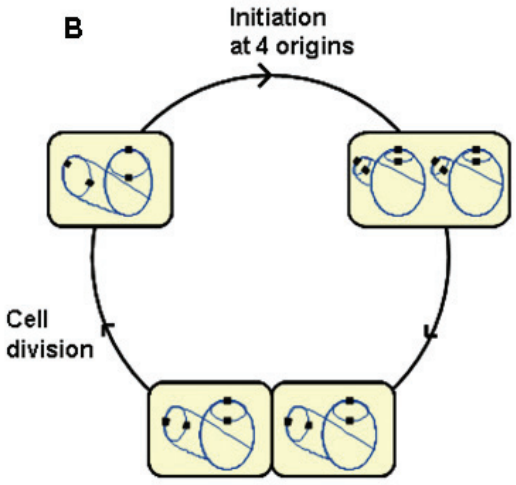

B
Initiation
at 4 origins
Cell
division

Fig 1

Replication pattern of *Escherichia coli* wild type cells.

Cells (yellow) with chromosomes (blue lines) and origins (black squares) are drawn schematically to show the number of replication forks and origins at different stages of the cell cycle during:

(A) Slow growth. The replication (C) and segregation (D) period is confined within one generation time ($T_d > C + D$). Initiation of replication occurs at one single origin and the chromosome is synthesized with a pair of replication forks.

(B) Rapid growth. The C + D period (1 ½ h) takes almost three generations (T_d ~½ h). Initiation of replication occurs at four origins early in the "grandmother generation". After initiation, DNA replication proceeds with twelve replication forks (eight new forks and four old forks) until cells division. A newborn cell has four origins and replicates with six replication forks. The figure was adapted from Paper III.

DNA replication

The complete sequence of genomic DNA of *E.coli*, which is a $4.7 * 10^6$ base pair circular double stranded molecule, has been reported (Blattner *et al.*, 1997). The *E.coli* genome is present as a highly compact structure in living cells. Replication of the bacterial genome is a prerequisite for subsequent cell division. The unique initiation site for DNA replication, *oriC*, is located at 84.1 minutes on the genetic map. One clockwise and one counterclockwise replication fork move bidirectionally from *oriC* towards the terminus, *ter*, on the opposite side of the chromosome. The fully replicated chromosome becomes decatenated and segregated to the coming daughter cells (Kornberg and Baker, 1992).

Initiation of replication from *oriC*

DNA replication is a highly regulated process and initiation occurs only once per cell cycle (Kornberg *et al.*, 1992). During rapid growth, the *oriC* region is present in several copies per cell. Initiation of multiple origins in rapidly growing cells occurs

simultaneously. Each cell will therefore at any time have 2^n origins (n = 0, 1, 2…). This is called initiation synchrony (Skarstad *et al.*, 1986)

The *oriC* region

The minimal *oriC* region consists of 245 base pairs and is composed of highly conserved regions proposed to be recognition sites for proteins interacting with the origin (Fig 2) (Oka *et al.*, 1980;Zyskind and Smith, 1986). Initial strand separation takes place in a region of helical instability containing three repeats of an AT-rich 13-mer sequence, 5′-GATCTNTTNTTTT-3, each starting with a GATC sequences (Bramhill and Kornberg, 1988;Kowalski and Eddy, 1989). Totally the minimal *oriC* region contains eleven GATC sequence (Zyskind *et al.*, 1986), which are recognition sites for Dam methylation (see page 11). The SeqA protein, necessary for inactivation of newly replicated origins, also interacts with the GATC sites in *oriC* (see page 12) (von Freiesleben *et al.*, 1994;Lu *et al.*, 1994;Slater *et al.*, 1995;Brendler *et al.*, 1995;Brendler and Austin, 1999). The AT-rich region of *oriC* contains three 6-mer binding sites (5′-AGATCT-3`) for the DnaA initiator protein (Speck *et al.*, 1999;Speck and Messer, 2001). DnaA also binds to five copies of the 9-mer consensus DnaA recognition sequence, 5′-TGTGNA$^{T}/_{A}$AA (Schaper and Messer, 1995), termed R boxes, (R1–R4) (Fuller *et al.*, 1984) and R5(M) (Matsui *et al.*, 1985), and to 9-mer I-sites (5`T$^{G}/_{T}$GGATCA$^{G}/_{A}$) (McGarry *et al.*, 2004). The I-sites and DnaA binding sites in the AT-rich region are low affinity sites and discriminate between the active form of DnaA, ATP-DnaA, and the inactive ADP-DnaA (Speck *et al.*, 1999;Speck *et al.*, 2001;McGarry *et al.*, 2004).

The *oriC* region do also contain binding sites for the architectural proteins IHF (Integration Host Factor) and Fis (Factor for inversion stimulation) (Leonard and Grimwade, 2005).

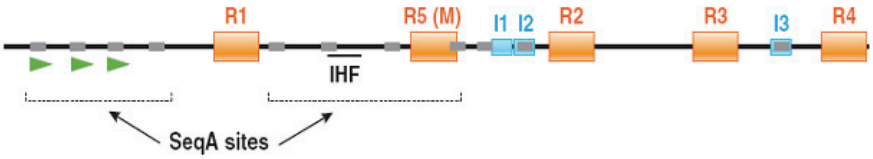


Fig 2

DNA sequence motifs in the *E. coli* chromosomal origin, *oriC.* The small gray bars represent GATC sequences recognized by Dam methylase and SeqA. Green arrowheads near the left border of *oriC* are 13-mer sequences that become single-stranded during open complex formation. The orange boxes correspond to DnaA binding sequences recognized by DnaA protein. Smaller blue boxes represent I-sites bound by ATP-DnaA. The site within *oriC* bound by integration host factor (IHF) is shown as a thin line between DnaA boxes R1 and R5 (M). The dashed lines represent two regions bound by SeqA protein. This figure was obtained from (Kaguni, 2006).

The DnaA initiator protein

The *dnaA* gene is located close to the *oriC* region and encodes the 52.5 kDa DnaA protein (Miki *et al.*, 1979;Skarstad and Boye, 1994). DnaA plays an essential role in the initiation of chromosomal replication (Kornberg *et al.*, 1992;Messer, 2002).

DnaA is an AAA^+ protein with structural similarities to archaeal and eukaryotic Orc1/Cdc6 proteins (Erzberger *et al.*, 2002). A comparison of primary sequences from various bacterial species and structural analysis suggests a subdivision of DnaA into four protein domains (Fig 3) (Fujita *et al.*, 1990;Messer *et al.*, 1999;Erzberger *et al.*, 2002). The conserved N-terminal domain I is involved in oligomerization of DnaA (Weigel *et al.*, 1999) and interaction with the DnaB helicase (Sutton *et al.*, 1998). Domain II of DnaA consists of a flexible linker, which differs widely in sequence and length among

DnaA proteins, and domain III contains the nucleotide binding region (a Rossman fold) (Schaper and Messer, 1997;Sutton and Kaguni, 1997). Domain III also contains Sensor I and II motifs of AAA+ family proteins (Koonin, 1992;Neuwald *et al.*, 1999). A membrane binding site is located between domain III and IV (Crooke *et al.*, 1992). The domain necessary for DNA binding is found close to the C-terminus (domain IV) (Roth and Messer, 1995;Fujikawa *et al.*, 2003).

The DnaA protein binds to ATP and ADP with high affinity, but only the ATP-DnaA is active in initiation of replication while ADP-DnaA is inactive (Sekimizu et al., 1987). The switch between active ATP-DnaA and inactive ADP-DnaA is important for regulation of initiation and further discussed in the General discussion section (see page 23).

The *E.coli* chromosome contains 308 chromosomal DnaA boxes in addition to the ones located in *oriC* (Roth and Messer, 1998). The *datA* locus contains five binding sites for DnaA and has an especially high capacity for DnaA binding (Kitagawa *et al.*, 1996;Kitagawa *et al.*, 1998). Titration of DnaA to *datA* was suggested to inhibit reinitiation of new replicated origins since deletion of the *datA* sequence resulted in overinitiation (Kitagawa *et al.*, 1998). Later overinitiation in Δ*datA* cells was found to be due to rifampicin-resistant initiations, suggesting that titration of DnaA to *datA* is not essential for restricting initiation to once per cell cycle (Morigen *et al.*, 2005). Binding sites for DnaA is also located in promoter regions of several genes (Messer and Weigel, 1997). The DnaA protein either activates, represses or terminates transcription of these genes dependent on the location of the DnaA binding sites in the promoters. The *dnaA* gene itself is repressed by binding of ATP-DnaA to DnaA boxes in the promoter regions of the gene. In contrast, ADP-DnaA protein represses the *dnaA* gene rather weakly (Speck *et al.*, 1999;Gon *et al.*, 2006).

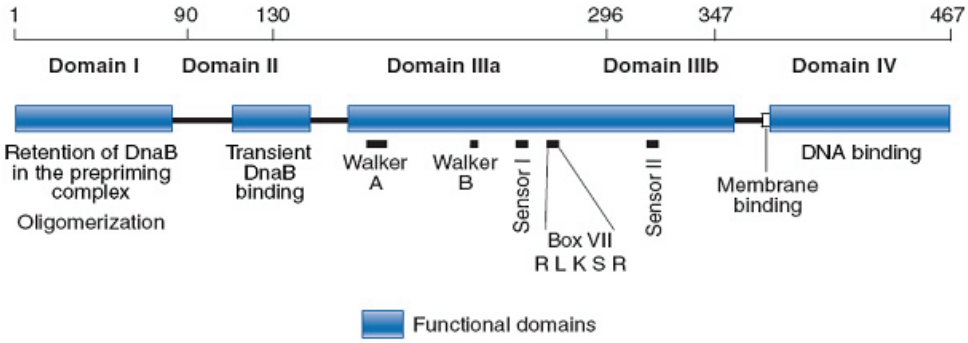


Fig 3

Functional domains of the DnaA protein. The different functional domains of the protein are indicated and discussed in the text. The scale at the top indicates the length in amino acids residues. The figure was obtained from (Kaguni, 2006).

Molecular events of initiation at *oriC*

Initiation of replication involves binding of several proteins to the minimal *oriC* region in a highly coordinated manner (Fig 4). The DnaA initiator protein, associated with either ATP or ADP, occupies the high affinity binding sites (R1, R2 and R4) in *oriC* throughout most of the cell cycle (Samitt *et al.*, 1989;Cassler *et al.*, 1995;Nievera *et al.*, 2006). Immediately prior to initiation of replication, binding of ATP-DnaA to the low affinity binding sites, leads to unwinding of the AT-rich region (Speck *et al.*, 2001;Leonard *et al.*, 2005). Several factors assist DnaA in DNA strand opening. The presence of negatively supercoiled template, transcriptional activation and the architectural proteins IHF or HU have a positive effect, whereas Fis affects duplex opening negatively (Dixon and

Kornberg, 1984;Skarstad *et al.*, 1990;Hwang and Kornberg, 1992;Asai *et al.*, 1992;Wold *et al.*, 1996).

The *oriC* bound DnaA direct a hexameric ring of the DnaB helicase in complex with six DnaC monomers, each of which bind one ATP (Funnell *et al.*, 1987;Baker *et al.*, 1987;Sekimizu *et al.*, 1988;Fang *et al.*, 1999). In complex with DnaC, the DnaB helicase activity is blocked. Release of DnaC is associated with ATP hydrolysis and activates bi-directional movement of DnaB helicase to open the DNA template (Wahle *et al.*, 1989). In the last stage of the initiation process, a physical interaction of DnaB with DnaG primase attracts the primase to the replication fork (Tougu *et al.*, 1994). DnaG synthesizes RNA primers to which DNA polymerase III holoenzyme binds. DNA polymerase III holoenzyme extends the primer ends and, assisted by DNA gyrase that releases topological stress, performs replication of the whole template (Kornberg *et al.*, 1992).

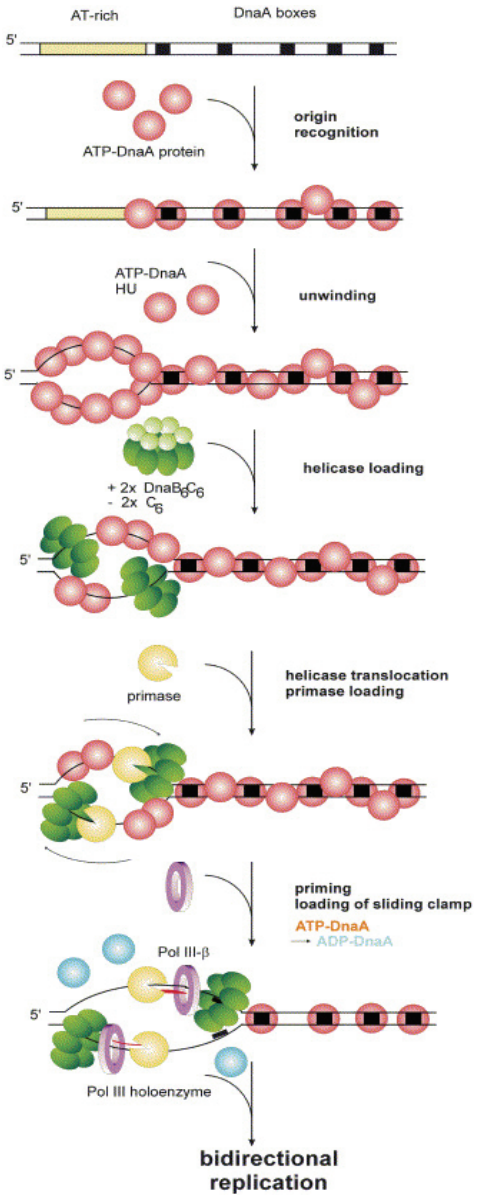


Fig 4

Molecular events of initiation at *oriC* in *E.coli*. The figure was obtained form (Messer, 2002).

Sequestration

Newly replicated origins are prevented from reinitiation through a process called sequestration (Campbell and Kleckner, 1990). The GATC sites are targets for DNA adenine metylatransferase (Dam methylase), which methylates the adenine residues at the N^6 position (Geier and Modrich, 1979). At the time of replication initiation, the GATC sites in *oriC* are fully methylated, i.e the adinine residue on each DNA strand is methylated. Semiconservative DNA replication generates hemimethylated DNA, where the old strand remains methylated and the new strand is unmethylated. The GATC sites in *oriC* remain hemimethylated for about one-third of the cell cycle, whereas a typical GATC site around the chromosome is remethylated immediately (Campbell *et al.*, 1990). A DNA sequence with an *oriC* like distribution of the GATC sites remain hemimethylated for the same period as *oriC* when located elsewhere on the chromosome (Bach and Skarstad, 2005).

Cells deficient in Dam (*dam*$^-$ cells) can not be transformed with fully methylated *oriC* plasmids (Messer *et al.*, 1985;Samitt *et al.*, 1989), presumably because the plasmids are sequestered by binding to the membrane in the hemimethylated state after one round of replication (Russell and Zinder, 1987;Ogden *et al.*, 1988;Landoulsi *et al.*, 1990). Hemimethylated origins are capable of undergo initiation of replication *in vitro* (Landoulsi *et al.*, 1989;Boye, 1991), but not *in vivo* (Russell *et al.*, 1987) arguing that some intracellular factor inactivates (sequesters) hemimethylated origins. To identify factors involved in sequestration, it was searched for mutants capable of transforming *oriC* plasmids in *dam*$^-$ background. All mutants had a defective *seqA* gene (von Freiesleben *et al.*, 1994;Lu *et al.*, 1994).

The SeqA protein

The *seqA* gene, encoding the 21 kDa SeqA protein, have homologues in the other *Enterobacteriaceae* species (Hiraga *et al.*, 2000). The *seqA* gene is non-essential and the protein appears in 1000 copies per cell (Slater *et al.*, 1995). SeqA has two functional domains, an N-terminal oligomerization domain (residues 1-50) and a C-terminal DNA binding domain (51-181) (Guarne *et al.*, 2002).

Biochemical analysis have shown that SeqA binds a minimum of two hemimethylated GATC sites and to fully methylated *oriC* (Slater *et al.*, 1995;Brendler *et al.*, 1995;Brendler *et al.*, 1999). Unmethylated *oriC* is not bound by SeqA (Slater *et al.*, 1995). The binding of SeqA to hemimethylated *oriC* suppress DNA melting and hence replication initiation *in vitro* (Lu *et al.*, 1994;Wold *et al.*, 1998;Torheim and Skarstad, 1999;Nievera *et al.*, 2006). *In vivo,* SeqA binding to hemimethylated origins is believed to be important for sequestration because *seqA* mutant cells (von Freiesleben *et al.*, 1994;Lu *et al.*, 1994) and cells in which eight of GATC sites in the origin are mutated to disrupted SeqA binding (Bach and Skarstad, 2004), initiate newly replicated origins more than once per cell cycle.

Other studies have suggested additional roles of the SeqA protein; contribution to correct folding of the chromosome (Torheim *et al.*, 1999;Weitao *et al.*, 1999;Klungsoyr and Skarstad, 2004), organization of newly replicated DNA (Hiraga *et al.*, 1998;Onogi *et al.*, 1999;Hiraga *et al.*, 2000) and proper chromosome segregation (von Freiesleben *et al.*, 2000;Bach *et al.*, 2003). All these subjects will be further discussed in the General Discussion section.

Elongation of replication

The coordinated action of many proteins is required to form a DNA replication fork to ensure an error-free and continuous DNA synthesis. The replication forks move around the circular chromosome at a rate of about 1000 nucleotides per seconds and meet about 40 minutes after initiation in a region opposite *oriC* (Kornberg *et al.*, 1992).

The replication fork includes two DNA polymerase III holoenzyme molecules (DNA pol III), DnaB helicase and DnaG primase. The activity of DnaB generates single-stranded DNA which is protected by single-stranded DNA binding protein (SSB). DNA gyrase relaxes the positive supercoils made by the helicase (Fig 5). DNA pol III moves in the 5`to 3` direction on one strand and in the 3`to 5`direction on the other stand. Because DNA pol III acts by adding deoxyribonucleotide triphosphates to the 3`-OH primer, only the strand complementary to the old 3` to 5` strand is synthesized continuously (leading strand). The new strand complementary to the old 5`to 3` strand is

synthesized by DNA pol III in series of relatively short pieces called Okazaki fragments (lagging strand). The RNA primers are replaced with DNA by the action of DNA polymerase I and Okazaki fragments are coupled with ligase (Kornberg *et al.*, 1992). Each molecule of the DNA pol III is associated with a ring-shaped sliding clamp, a dimer of the β-subunit of DNA pol III (β-clamp). The β-clamp encircles the DNA strand (Kong *et al.*, 1992;Kelman and O'Donnell, 1995) and stabilizes the DNA polIII-DNA association. The β-clamp is loaded onto DNA by the clamp loader, the γ-complex of DNA pol III (Stukenberg *et al.*, 1991;Katayama, 2001).

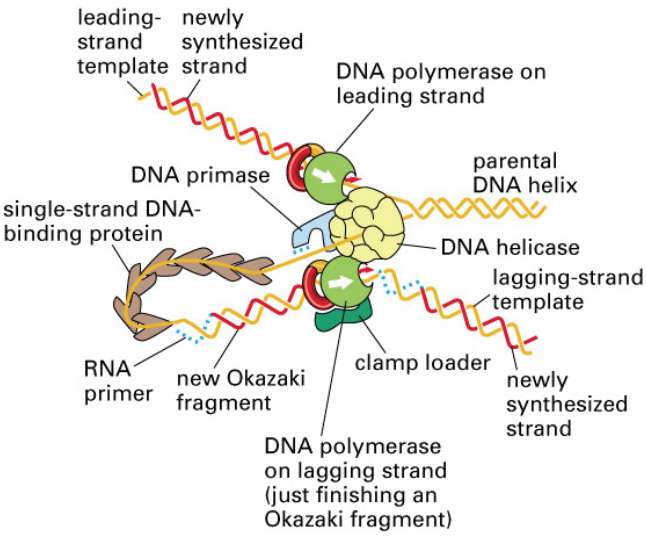


Fig 5

The replication fork. Proteins at the replication fork are indicated. The figure was obtained from Molecular Biology of the Cell, 4th Edition.

Termination of replication

A cycle of DNA replication in *Escherichia coli* ends when the replication forks converge on the opposite side of the chromosome in a region called the terminus (generically called *ter*). The *ter* region is composed of two sets of inverted repeats that allow replication forks to enter the terminus but not exit (Hill *et al.*, 1987;Hill *et al.*, 1988b). Function of the *ter* sites is dependent on the presence of a trans-acting factor specified by the *tus* locus (Hill *et al.*, 1988a;Hill and Marians, 1990). The Tus-t*er* complex acts to block the action of the replicative helicase, DnaB, resulting in termination of DNA replication (Neylon *et al.*, 2005). The termination of replication precedes the physical separation of the newly duplicated sister chromosomes prior to cell division.

Chromosome segregation and cell division

The products of circular replication are topologically linked/ catenated. Homologous recombination between sister chromosomes also often causes a knotted dimer. Such structures must be resolved into monomeric chromosomes prior to segregation. The replication products are decatenated by topoisomerase IV (Kato *et al.*, 1990), which is composed of a heterotetramer formed by a ParE dimer (with an ATPase domain) and a ParC dimer (with a DNA binding and cleavage domain) (Peng and Marians, 1993).The XerC/ XerD recombinase, in conjugation with FtsK protein, is responsible for resolving knotted dimers at the *dif* site in the terminus (Steiner and Keumpel, 1998; Steiner et al., 1999).

Cell division involves partitioning of the cytoplasm into two compartments, each containing one copy of the cell`s genetic information. The site of cell division is placed with high fidelity at the mid-cell position prior to cell division and is accomplished by the action of the MinCDE system. The MinCDE system undergoes an oscillation cycle, in which the structures disassemble at one pole and then undergo another assembly and disassembly at the opposite side of the cell. As a result, division is prevented at the end of the cell and only allowed at sites near mid-cell (Rothfield *et al.*, 2005).

FtsZ is a tubulin-like GTPase that is essential in cell division. FtsZ forms a circumferential ring on the inside of the cytoplasmic membrane at the site of division called the Z ring. The Z ring is probably placed at mid-cell by a combination of nucleoid occlusion that prevents septum formation at positions occupied by the nuleoids (Mulder and Woldringh, 1989;Yu and Margolin, 1999) and prevention of septum formation at the cell poles by the MinCDE system (see above). The presence of the Z ring is a prerequisite for assembly of at least ten other division proteins into a multiprotein complex to perform cell division (Rothfield *et al.*, 2005).

Aims of the present work

The objective of the present work has been to gain further insight into the timing and coordination of DNA replication in *Escherichia coli*. We particularly wanted to:

Paper I and II

Understand more about the role of the wild type SeqA protein in initiation of replication by physiological and biochemical studies of two sequestration deficient mutants, *seqA2* and *seqA4*. The SeqA2 mutant protein contains a single amino change in the C-terminal region of the protein, whereas the SeqA4 mutant protein contains a single amino change in the N-terminal region.

Paper III

Study dynamic organization of sister origins and replication forks during multifork DNA replication and investigate the possible involvement of sequestration by SeqA in this process.

Paper IV

Develop a novel, cell-based positive screening assay for the discovery of new antibiotics targeting the DNA replication initiator protein, DnaA.

Summary of results

Paper I

The SeqA protein was identified in a screen for mutants that were unable to sequester newly replicated, hemimethylated origins. In this work one of these mutants, *seqA2*, harbouring a single amino acid change in the C-terminal end of the SeqA protein (N152D) was further characterized. We found that the SeqA2 protein was not able to support formation of discrete SeqA foci *in vivo*. The SeqA foci observed in wild type cells are believed to arise from multimerization of SeqA on hemimethylated DNA at the replication fork, presumably representing organization of the newly formed DNA. *In vitro,* we found that the SeqA2 protein was less efficient in hemimethylated DNA binding compared to the wild type protein. SeqA2 was able to interact with hemimethylated *oriC* at 100 times higher concentrations than the wild type protein. However, a characteristic ladder like pattern that was observed upon binding of wild type SeqA to the *oriC* fragment was not formed upon binding of SeqA2. This indicated that the SeqA2 protein also experienced defects in protein multimerization. *In vitro* studies indicated that SeqA2 made a multimer that was different from the wild type multimer. We therefore suggested that highly ordered multimerization of SeqA and hemimethylated origin binding is necessary for sequestration and in the process of organizing newly replicated DNA.

Paper II

In Paper I we studied a sequestration deficient mutant with a mutation in the C-terminal domain. In this work, another sequestration deficient mutant, *seqA4*, containing a single amino acid change in the N-terminal domain (A25T) was studied. We found that the SeqA4 mutant protein had lost its ability to form higher-order multimers, but was capable of binding as a dimer with wild type affinity to a pair of hemimethylated GATC sites. *In vitro,* we found that both SeqA and SeqA4 dimers were able to generate positive supercoils when bound to DNA. In contrast, only wild type SeqA was able to form a higher-order structure that lead to a change of the DNA topology in the "opposite" direction, resulting in restraint of negative supercoils.

In vivo, excess SeqA4 protein was able to restore initiation synchrony, but not able to support formation of discrete SeqA foci at the replication fork. We suggested that origin sequestration is dependent on extensive binding of SeqA to GATC sites, whereas organization of newly replicated DNA at the replication fork requires higher-order multimerization of SeqA.

Paper III

Organization of the DNA replication machinery into one unit, the replication factory, was proposed by Dingman (Dingman, 1974). He also suggested that newly replicated DNA moves away from the anchored replication machinery as replication proceeds. In paper III we studied origin and replisome organization in rapidly growing cells containing multifork replication cycles. We found that sister origins were colocalized for 1-2 generations during rapid growth when initiation occurred in the "grandmother" or "mother" generation. We also found that the degree of origin colocalization decreased at slower growth rates. The extensive colocalization of the origin region during rapid growth might be a mechanism to ensure that the correct pair of chromosomes is segregated, since each of the segregating chromosomes contains several forks and two or four origins. We found that also sister replication forks were extensively colocalized during rapid growth. The role of origin sequestration by SeqA in origin and replication fork colocalization was assessed. We found that colocalization of new origins and sister replication forks was independent of origin sequestration, but dependent on the presence of functional SeqA. *In vitro* experiments suggested that SeqA is capable of pairing newly replicated sister DNA molecules.

Paper IV

The conditional mutant *dnaA219*(Cos) has a cold sensitive phenotype (Weigel *et al.*, 1999). The *dnaA219* mutant dies at lower temperatures (30°C) due to too much DnaA219 activity and therefore excessive DNA synthesis relative to cell mass. At higher temperatures (42°C) growth is obtained probably due to a partially inactive DnaA219 protein. We have utilized the properties of the *dnaA219* mutant to develop a screen for novel antibiotics targeting the DnaA protein. In this screen a potential drug will be discovered by the reduction of DnaA219 overactivity and thus recovery of growth at the non-permissive temperature (30°C). We prepared a test strain that could initiate DNA replication independent of *oriC* and DnaA, and therefore allow testing of only one concentration of each substance. The resulting strain SF53 (*dnaA219rnhA*) grew at 42°C, but died at 30°C. We found that SF53 survived at 30°C if the N-terminal 86 amino acids (domain I), important for oligomerization and formation of proper initiation complexes, of wild type DnaA were independently produced. This is probably due to formation of inactive hetero-oligomers of DnaA and domain I, leading to a poisoning of the *oriC*-DnaA initiation complex (Weigel *et al.*, 1999). Furthermore, we found that SF53 assay transfer into high-throughput format was successful. We calculated a Z-factor of 0.55, meaning that the assay lies within the category of "excellent assays" (Zheng *et al.*, 2001). However, when SF53 was tested in a pilot study with 4199 microbial extracts, we were not able to identify any extract with a compound targeting DnaA.

General discussion

Regulatory mechanisms in DNA replication initiation

Replication of the chromosome is a central event in the bacterial cell cycle. Initiation is precisely timed in the cell cycle, each origin is initiated only once per cell cycle and all origins in the same cell are initiated simultaneously (Skarstad *et al.*, 1986). There is a fine balance between the positive and negative factors that are involved in the regulation of initiation of chromosome replication.

Timing of replication initiation

Donachie noticed in 1968 that initiation of DNA replication occurs when a certain mass per chromosomal origin, the initiation mass, is reached (Donachie, 1968). The initiation mass varies somewhat with growth rate (Wold *et al.*, 1994), meaning that another factor is important to define the time of initiation. The DnaA protein was suggested to be the element that determines the initiation mass. Increased levels of DnaA leads to initiation at a lower cell mass (Atlung *et al.*, 1987;Skarstad *et al.*, 1989;Lobner-Olesen *et al.*, 1989) and titration of DnaA away from *oriC* by introduction of extra DnaA titrating *datA* sites increased the initiation mass (Morigen *et al.*, 2003). Several models have emerged to explain the observed constancy of the initiation mass. “The inhibitor dilution model” suggests that an inhibitor is synthesized at initiation of replication to inhibit reinitiation (Pritchard *et al.*, 1969). As the cell grows, the concentration of the inhibitor drops to a concentration that allows initiation of replication to occur. Another model, “the autorepressor model” suggests that as the cell grows an initiator is synthesized and will eventually reach a level high enough for initiation to occur (Sompayrac and Maaloe, 1973). The models were combined and modified into “the initiator titration model” where DnaA is suggested to be the initiator (Hansen *et al.*, 1991b). This model suggests that accumulation of a critical amount of DnaA protein leads to an initiation event. The level of free DnaA is regulated by titration of DnaA to high affinity DnaA boxes around the chromosome (Hansen *et al.*, 1991b). *oriC* has some low affinity DnaA binding sites, thus the DnaA boxes in the origin is not filled with enough DnaA to initiate replication before other high affinity binding sites around the chromosome are filled (Hansen *et al.*, 1991a).

After initiation of replication the bound DnaA is assumed to be released and may be reused in other origins present in the same cell and trigger new initiation (Lobner-Olesen *et al.*, 1994), which results in an almost simultaneous initiation on multiple origins during fast growth (Skarstad *et al.*, 1986).

Once per cell cycle replication

Several mechanisms contribute to ensure that initiation of replication occurs only once per cell cycle and are discussed below.

Sequestration of the *oriC* region

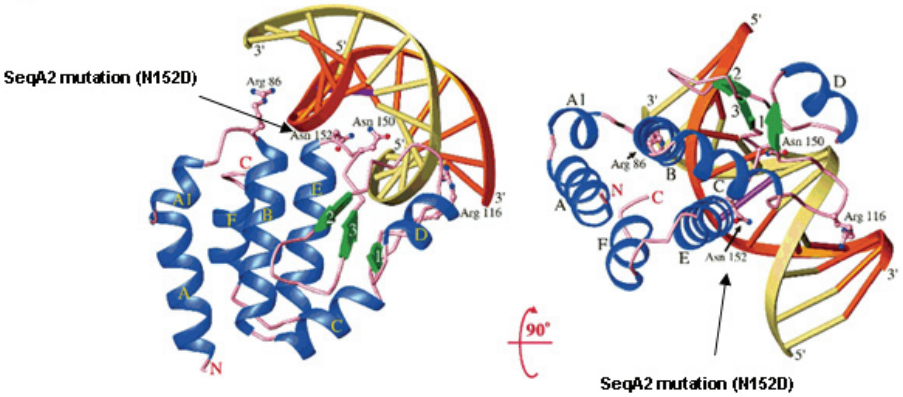
Sequestration of newly replicated, hemimethylated origins renders the origin inaccessible for a new round of replication. As mentioned in the Introduction section (page 12), the SeqA protein plays a key role in this process. Von Freiesleben and coworkers isolated *seqA* mutant strains that were capable of replicating fully methylated *oriC* plasmids in *dam*$^-$ background (von Freiesleben *et al.*, 1994). We have studied the physiological and biochemical properties of two of the mutants, *seqA2* and *seqA4* (Paper I and II). The SeqA2 mutant protein contains a single amino acid change in the C-terminal domain of the protein, in which an Asn is changed to an Asp at position 152 (N152D) (Paper I). SeqA2 was not capable of binding to a hemimethylated *oriC* fragment *in vitro*. Later structural studies revealed that the C-terminal domain is responsible for DNA binding. The SeqA C-terminal interacts with DNA mainly in the major groove of the hemimethylated GATC sequence (Fig 6A) (Guarne *et al.*, 2002). The crystal structure also shows that the Asn 152 forms close van der Waals contacts and direct hydrogen bonds with the A-T base pair (Guarne *et al.*, 2002) and was probably disrupted upon DNA binding of the SeqA2 protein. Association of SeqA and hemimethylated DNA is highly cooperative leading to formation of large complexes (Slater *et al.*, 1995;Kang *et al.*, 1999;Skarstad *et al.*, 2000;Lee *et al.*, 2001). We found that the ability of purified SeqA2 to form higher-order multimers *in vitro* was altered compared to the wild type protein (Paper I). *In vivo*, the SeqA2 protein was completely inactive in sequestration and even a 20-fold overproduction of SeqA2 did not support initiation synchrony. On the

other hand, when SeqA2 was overproduced in a SeqA wild type background, initiation synchrony was affected. The interaction of SeqA2 with wild type protein might result in formation of a mixed oligomer that is unable to bind hemimethylated origins and thereby unable to prevent reinitiation from *oriC*. From these results we suggested that formation of higher-order multimers and correct binding of these to hemimethylated DNA is necessary for origin sequestration (Paper I).

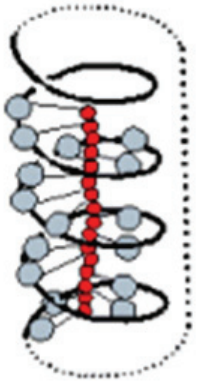
The SeqA4 mutant protein contains a single amino acid change in the N-terminal domain of the protein, in which an Ala is changed to a Thr in position 25 (A25T) (Paper I). SeqA4 was incapable for forming higher-order multimers *in vitro*. However, the mutant protein was capable of forming a dimer which was suggested to be the basic binding unit (Paper II). This was supported by structural analysis of SeqA N-terminal that revealed that the structural unit of SeqA is a dimer (Guarne *et al.*, 2005). A SeqA4 dimer bound with wild type affinity to a pair of hemimethylated GATC sites *in vitro* and excess SeqA4 protein led to partially restored initiation synchrony *in vivo* (Paper II). The reason for this might be that origin sequestration does not require a perfect higher-order SeqA multimer per se, but does require a high local concentration of SeqA. In contrast, high local concentration of SeqA2 did not restore initiation synchrony (Paper I). This might be mainly explained by the lack of binding to hemimethylated GATC sites.

Sequestration of the *oriC* region lasts for about one-third of the cell cycle (Campbell *et al.*, 1990). When sequestration has ended, the newly replicated origins become accessible for new rounds of initiation. Therefore additional mechanisms must ensure that the initiation potential is lowered during sequestration. This is discussed below.

A


SeqA2 mutation (N152D)
Arg 86
Asn 152
Asn 150
Arg 116
90°
SeqA2 mutation (N152D)

B



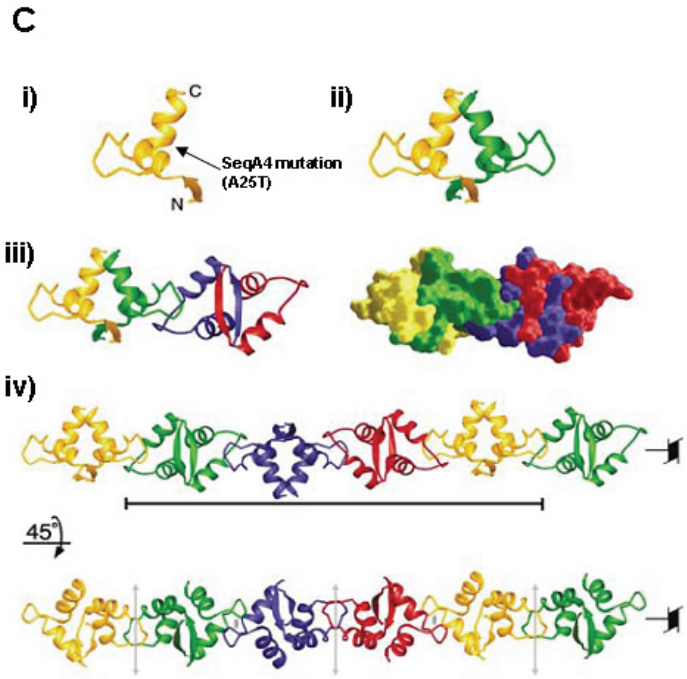


Fig 6

(A) Structure of the SeqA-C−DNA complex. Ribbon diagrams of SeqA-C bound to the 12 base pair hemimethylated DNA in two orthogonal views. α-helices are blue; β-strands, green. The unmethylated strand is yellow, and the methylated one is orange. DNA bases are shown as thin rods, and the methylated adenine is purple. The single amino acid change in the SeqA2 mutant protein (N152D) is indicated with arrows. The figure was obtained from (Guarne et al., 2002).

(B) A model of a left-handed SeqA multimer with the negatively supercoiled DNA (black line) wrapped around the SeqA filament (grey and red circles). The figure was obtained from Paper II.

(C) Oligomerization of the SeqA–N dimer. (i) Ribbon diagram of a single SeqA–N subunit. The single amino acid change in the SeqA4 mutant protein (A25T) is indicated with an arrow (ii) A SeqA–N dimer. The two subunits are shown as yellow and green (iii) The asymmetric unit contains two SeqA–N dimers (iv) Two views of the SeqA–N filament. The black bar indicates a complete helical turn consisting of four dimers. This figure was obtained from (Guarne et al., 2005).

Sequestration of the *dnaA* gene promoter

Expression of the *dnaA* gene is greatly reduced during the period of sequestration (Campbell *et al.*, 1990). The central part of the *dnaA* promoter contains six GATC sequences. Methylation of these sites is required for maximum promoter activity (Braun and Wright, 1986;Kucherer *et al.*, 1986). One of these sites, located within the -35 region of the *dnaAp2* promoter, remained hemimethylated for about the same period as the GATC sites within the *oriC* region. This implies that that the *dnaA* promoter region is subject to sequestration analogous to that observed at *oriC* (Campbell *et al.*, 1990). Recently, *in vivo* studies revealed that coordinated replication and sequestration of *oriC* and *dnaA* were required for maintaining controlled once per cell cycle initiation of chromosome replication (Riber and Lobner-Olesen, 2005). The *dnaA* gene was moved to another location on the chromosome so that sequestration of the *dnaA* promoter did not coincide with origin sequestration. This resulted in increased availability of the DnaA protein towards the end of the origin sequestration period and thus reinitiation of newly replicated origins.

Regulation of DnaA activity

The level of ATP-DnaA increases towards the time of replication initiation (Kurokawa *et al.*, 1999). The increased level of ATP-DnaA originates from a membrane dependent exchange of ADP for ATP and increased transcription of the *dnaA* gene (newly made DnaA is in the ATP form) (Crooke *et al.*, 1992;Boeneman and Crooke, 2005).

Immediately after replication initiation hydrolysis of active ATP-DnaA to inactive ADP-DnaA occurs. This is a regulatory inactivation of DnaA (RIDA) to prevent reinitiation of an already initiated origin (Boye *et al.*, 2000;Kato and Katayama, 2001). Inactivation of DnaA by RIDA requires the β-clamp to be loaded onto DNA, the presence of a partially purified factor, IdaB (Katayama *et al.*, 1998), and is stimulated by ongoing DNA replication (Kurokawa *et al.*, 1998). The Hda protein, necessary for proper regulation of initiation (Camara *et al.*, 2003;Riber *et al.*, 2006), has later been shown to possess IdaB activity (Su'etsugu *et al.*, 2005).

Mutations in DnaA that affect binding or hydrolysis of ATP may induce the protein to be constitutively active, leading to overinitiation (Kellenberger-Gujer *et al.*, 1978;Katayama and Kornberg, 1994). One such mutant, *dnaAcos*, is a cold-sensitive spontaneous revertant from the temperature-sensitive *dnaA46* mutant (Kellenberger-Gujer *et al.*, 1978) and contains the mutations A184V and H252Y (from *dnaA46*) and Q156L and Y217H (Braun *et al.*, 1987;Hansen *et al.*, 1992). The DnaAcos protein has been purified and characterized *in vitro*. The mutant protein binds to DNA containing the origin region and functions in the loading of DnaB helicase onto single-stranded DNA, but is unable to bind nucleotide (Katayama *et al.*, 1995). DnaAcos seems to be an unregulated form of DnaA protein that is always active in initiation and therefore causes excess DNA replication at lower temperatures (30°C). At higher temperatures (42°C) the protein is apparently partially inactive, explaining that the overinitiation is reduced (Katayama, 1994;Katayama *et al.*, 1994).

The phenomenon of lethal overactivity of DnaA was utilized to develop a screen for discovery of novel antibiotics targeting DnaA (Paper IV). A cold-sensitive *dnaA* mutant, with properties that closely resemble the properties of the *dnaAcos* mutant from Kellenberger-Gujer et al., 1978, was isolated (Weigel et al., 1999). The mutant strain contains the *dnaA46* mutations (see above) and a novel *dnaA* allele, designated *dnaA219*(Cos) (R342C). The idea of the screen was to search for drugs that will inactivate DnaA219 and therefore allow growth at the non-permissive temperature (30^{o}C). *dnaA219* was improved and made amenable for high-throughput screening by introduction of an alternative initiation pathway by deletion of the *rnhA* gene, encoding RNaseHI, thus rendering initiation independent of *oriC* and DnaA (Kogoma, 1997). The

assay transfer of *dnaA219rnhA* into high-throughput format was successful, but in a pilot study of about 4000 extracts no compound that inhibit DnaA activity was found (Paper IV). There are several possible reasons for this. The microbial extracts may not contain any components targeting DnaA or the extracts might contain a DnaA inhibitor, but growth is not obtained due to the presence of second antibiotic. A third possibility is that relevant compounds were not able to enter the cell.

Most antibiotics known today target the bacterial cell wall, the protein synthesis apparatus or DNA topoisomerases (Gale *et al.*, 1981;Amabile-Cuevas *et al.*, 1995;Walsh, 2003). So far no drug targeting the essential DNA replication machinery has been discovered. The DnaA protein is highly conserved among bacteria (Weigel and Messer, 2002) and necessary for DNA replication. Therefore, discovery of a compound that inactivates DnaA would be a novel mechanism in the treatment of infectious diseases.

Discovery of a compound that specifically inactivates DnaA would also be a great advantage in studies of DNA replication control mechanisms. Such a compound could increase the understanding of the physiological and biochemical properties of DnaA similar to the understanding of gene transcription by RNA polymerase. RNA polymerase is specifically inactivated by rifampicin (Messer, 1972;Lark, 1972;Zyskind *et al.*, 1977).

Chromosome organization and segregation

A central problem in biology is faithful transmission of hereditary information from the mother to its daughter cells. This process involves precise organization and partitioning of the newly synthesized sister chromosomes. The newly synthesized sister chromosomes have to be organized and condensed to ensure proper segregation. The bacterial chromosome is organized into a highly compact structure. *In vivo*, DNA topoisomerases maintain the bacterial chromosome in a defined underwound state, which is essential for proper functioning of all cellular processes that require DNA strand separation, such as DNA replication, transcription, recombination and repair. DNA topoisomerases were the first proteins identified to have a role in chromosome partitioning. They are not directly involved in chromosome partitioning, but have a role in the production of substrates that are viable for segregation (Draper and Gober, 2002).

Other proteins probably involved in organization and proper segregation of newly replicated DNA is MukB, a protein similar to eukaryote and Gram-positive SMC (Stable Maintenance of Chromosome) proteins (Niki *et al.*, 1991;Niki *et al.*, 1992) and small nucleoid associated proteins such as HU (histone-like protein) and H-NS (histone-like nucleoid structuring protein) (Sherratt, 2003). *mukB* mutant cells produce a lot of anucleate cells (Niki *et al.*, 1991). MukB acts in concert with MukE and MukF and has the structure of a motor protein (Niki *et al.*, 1991;Niki *et al.*, 1992;Yamanaka *et al.*, 1996). MukB binds unspecifically to DNA and is probably not directly involved in segregation. However, the nucleoid is more dispersed than the wild type nucleoid (Weitao *et al.*, 1999), suggesting a role in chromosome condensation. Recently an intergral inner membrane protein, SetB, was found to strongly affect chromosome organization and segregation (Espeli *et al.*, 2003). SetB localizes in the cell as a helix and interacts with MreB, the bacterial actin homologue, which also forms a helix (Espeli *et al.*, 2003;Kruse *et al.*, 2003). These observations suggest that there may be a link between chromosome segregation and cellular infrastructure.

The SeqA protein also seems to be important for organization and segregation of the chromosome. *seqA* mutant cells and cells with excess SeqA protein show aberrant nucleoid folding and segregation *in vivo* (Lu *et al.*, 1994;Weitao *et al.*, 1999;von Freiesleben *et al.*, 2000;Bach *et al.*, 2003). In addition, the SeqA protein was found to affect the DNA topology *in vitro* (Torheim *et al.*, 1999;Klungsoyr *et al.*, 2004). Formation of discrete SeqA foci in wild type cells are believed to arise from multimerization of SeqA on hemimethylated DNA at the replication fork, presumably representing organization of newly formed DNA by SeqA (Hiraga *et al.*, 1998;Onogi *et al.*, 1999;Hiraga *et al.*, 2000).

The SeqA2 mutant protein was unable to restrain negative supercoils *in vitro* and form discrete SeqA foci *in vivo*. These defects might be explained by the inactivity of SeqA2 in correct and stable binding to hemimethylated DNA (Paper I). SeqA4 was able to form a dimer that bound hemimethylated DNA, but failed to form higher-order multimers (see page 22) (Paper II). Binding of SeqA4 dimers to DNA led to a topology change equivalent to positive supercoiling. In contrast, the topology change that resulted in restraint of negative supercoils could not be made by the SeqA4 protein. We suggested

that for this type of topology change, binding of single, independent dimers is not sufficient, and formation of a SeqA multimer is required. A model was designed to explain the finding that negative supercoils of DNA bound to a SeqA multimer become restrained (Fig 6B) (Paper II). In this model SeqA forms a left handed helix with the negatively supercoiled DNA wrapped around the SeqA filament. The model was supported by structure determination and modeling of the SeqA N-terminus, which indicates that it forms a helical fiber made up of dimers (Fig 6C) (Guarne *et al.*, 2005). The SeqA4 protein was also unable to form discrete SeqA foci *in vivo*. This indicates that specific N-terminus interactions are required for generating such structures. It is probable that the foci consist of helical SeqA filaments bound to hemimethylated DNA (Paper II).

Eukaryotic chromosomes have a locus, the centromere, at which force is applied to separate replicated chromosomes. In bacteria, the replicon theory proposed that chromosome segregation is a passive mechanism coupled to membrane growth via attachment of specific chromosomal sites to the cell membrane (Jacob *et al.*, 1963). Later is has been suggested that sites close to the origin region of the chromosome carry out the centromere function in *E.coli*. The hemimethylated *oriC* could be a candidate (Ogden *et al.*, 1988). However, deletion of *oriC* does not affect the localization pattern of the region surrounding *oriC* (Gordon *et al.*, 1997;Berkmen and Grossman, 2007) and plasmids containing *oriC* do not localize in a discrete fashion inside the cell (Niki and Hiraga, 1999). In *E.coli* the *cis*-acting sequence, *migS*, was recently discovered to be involved in active segregation of the chromosome (Yamaichi and Niki, 2004;Fekete and Chattoraj, 2005). The 25 base pair *migS* sequence is located close to the replication origin and is supposed to play a crucial role in bipolar positioning of *oriC* (Yamaichi *et al.*, 2004).

Dynamic organization of E.coli *DNA replication cycle components*

Formerly it was proposed that the chromosome and protein components of the bacterial cell cycle were highly unorganized and that movement mostly relied on physical forces and self-organizing mechanisms. In the last ten years, new fluorescence microscopy techniques have revealed specific organization of cellular processes within the bacterial cell. Positioning and proper organization of the DNA replication cycle components may contribute to timely regulation of the replication initiation, orderly separation of daughter chromosomes and efficient DNA replication (Thanbichler and Shapiro, 2006). Several models have tried to explain how the replication machinery and chromosome is organized in the cell. The models are based on different types of microscopy techniques and involve growth of cells at different rates. The models therefore have some conflicting conclusions, which are discussed below.

The Replication Factory model

Organization of the replication machinery into one unit, "the Replication Factory", containing both forks originating from the same origin was initially proposed by Dingman (Dingman, 1974). In this model the replication machinery is tethered to the cell centre and the chromosome feeds into it as replication proceeds. Initially the model got support from cytological studies of replication fork localization in live *B.subtilis* cells (Lemon and Grossman, 1998;Lemon and Grossman, 2000). The replication fork was visualized with GFP-tagged replication protein. The factory model has also got support from studies of fixed *E.coli* cells in which newly replicated DNA was visualized with immunostaining of SeqA (Hiraga *et al.*, 1998;Molina and Skarstad, 2004) or by pulse-labeling with [$^{(3)}$H] thymidine followed by electron-microscopic autoradiography (Koppes *et al.*, 1999). In another study of replication fork localization using GFP-tagged SeqA protein evidence for associated replication forks was not found. The authors reported a 1:1 correspondence between replication forks and SeqA foci (Brendler *et al.*, 2000). The existence of replication factories is therefore still controversial.

In paper I and III we studied replication fork colocalization during rapid growth. The replication fork was visualized by immunostaining of SeqA (Paper I) or by BrdU labeling and subsequent immunostaining (Paper III). The number of replication fork foci was compared with the number of origins and replication forks as determined by flow cytometry analysis of the same cells. In paper I we found that the number of replication fork foci was about half the number of origins per cell in two different wild type strains. This indicates that two or more replication forks are held together forming one replication fork focus. In paper III we found that the distributions of BrdU foci were in agreement with the distributions of foci found by immunostaining of SeqA (Hiraga *et al.*, 1998;Molina *et al.*, 2004) (Paper I). The average number of forks per cell was calculated from flow cytometry histograms and found to be about twice the average number of replication fork foci per cell. Again we found that pairs of replication forks were colocalized throughout most of the cell cycle. Both studies therefore supports the replication factory model (Dingman, 1974;Lemon *et al.*, 1998). In a study of rapidly growing cells it was also found that immediately after initiation, the eight or twelve replication forks were organized into a higher-order structure (Molina *et al.*, 2004).

Recently the existence of the replication factory has got support from studies in budding yeast. It was found that sister replication forks generated from the same origin stay associated with each other within a replication factory during replication (Kitamura *et al.*, 2006).

The Translocating Replication Apparatus model

Hiraga and coworkers have suggested that the Replication factory model needs some modifications (Hiraga *et al.*, 2000). In the Translocating Replication Apparatus model the *oriC* region is localized at mid-cell prior to replication and a pair of replication forks will form at the same site upon initiation of replication. As replication proceeds, a SeqA multimer will bind to nascent, hemimethylated DNA to form a SeqA focus. The SeqA focus then separates into two foci that migrate in opposite directions to the ¼ and ¾ positions. The sister *oriC* copies however, stayed associated at mid-cell. After release of cohesion, the sister chromosomes rearranged to form separate nucleiods that moved

towards the cell pole. It was suggested that the migration is due to an active segregation mechanism (Hiraga, 2000;Hiraga *et al.*, 2000).

The Extrusion-Capture model

Dingman also proposed that newly replicated DNA moves away from the anchored replication machinery as replication proceeds. This proposal was refined and named as the Extrusion-Capture model (Lemon and Grossman, 2001). In this model the newly replicated chromosome is directed away from the cell centre towards the cell poles by the replication process itself. Thus, segregation of sister chromosomes occurs as replication proceeds (Sawitzke and Austin, 2001;Lemon *et al.*, 2001). This model has got support from localization studies of chromosomal loci, which have been detected with fluorescence in fixed cells by *in situ* hybridization (FISH) (Niki and Hiraga, 1998) or by using fluorescent techniques such as GFP-ParB/*parS* system (Li *et al.*, 2002;Nielsen *et al.*, 2006) or the GFP-LacI/*lac* operator system (Gordon *et al.*, 1997;Lau *et al.*, 2003;Wang *et al.*, 2005;Berkmen and Grossman, 2006). In slowly growing cells, in which DNA replication is confined to a single division cycle (Fig 1A). The sister origins region were found to stay in close proximity at mid-cell for a short period following initiation before they separated and moved towards the future mid-cell positions (Wang *et al.*, 2005;Bates and Kleckner, 2005;Nielsen *et al.*, 2006). Furthermore, the chromosomal loci were found to segregate according to their distance from the origin (Nielsen *et al.*, 2006).

The Sister Chromosome Cohesion model

Distinct from the Factory model by Dingman, evidence has been presented that replication produces sister chromosomes that are paired during much of their replication period and later separate in a coordinated manner (Niki *et al.*, 1999;Hiraga *et al.*, 2000;Niki *et al.*, 2000;Sunako *et al.*, 2001;Bates *et al.*, 2005). Interpretation of some of these results may not be straightforward due to the use of synchronized mutant cells, since such cells has been reported to be altered in replisome organization, and therefore

also maybe in chromosome organization, compared with wild type cells (Bach *et al.*, 2003).

In Paper III we studied origin organization in rapidly growing *E. coli* wild type cells using the GFP-LacI/*lac* operator system. The cells initiated at two or four origins and therefore replicated with multiple replication cycles. We found in cells that initiated replication towards the end of the "grandmother" generation or early in the "mother" generation, new origins were spatially localized into a structure that could not be visually separated within the resolution of the light microscope for most of the cell cycle (Paper III). In slowly growing cells chromosome segregation was found to start within a short time-period after replication initiation and does not wait till completion of replication (see above). Since we found extensive colocalization of origins with multifork DNA replication during rapid growth, we decided to investigate how the organization of origins may change as cellular growth rate varies. We found that the period of origin colocalization increased with decreasing growth rate (Paper III). During rapid growth the replication pattern is more complex with the two segregating chromosomes having several replication forks and two or four origins (Fig 1B). These cells have to ensure that the "correct" pair of chromosomes segregates. We suggested that colocalization during rapid growth is a way to organize the chromosome preventing the cell from segregating the incorrect DNA strands (Paper III).

The role of SeqA in sister origin and replisome organization

We found extensive colocalization of sister origins and replication forks during rapid growth (see above). The role of origin sequestration by SeqA in this colocalization was investigated. The studies were performed using two different mutant cells. In the *oriCm3* mutant cells, eight of the origin GATC sites have been changed to GTTC (Bach *et al.*, 2004). The origins of these cells cannot bind SeqA, and therefore cannot be sequestered. The other type of mutant cells have an in-frame deletion in the *seqA* gene (Slater *et al.*, 1995). These cells are unable to sequester newly replicated origins and also deficient in SeqA binding of newly replicated, hemimethylated, chromosomal DNA in general (von

Freiesleben *et al.*, 1994;Lu *et al.*, 1994;Slater *et al.*, 1995). We found wild type organization of new origins and sister replication forks in the *oriCm3* mutant cells. In contrast, the cells without SeqA were large and had multiple origin and replication fork foci. The number of origin foci and replication fork foci seemed to roughly equal the number of origins and replication forks as measured with flow cytometry, respectively. These results indicated that colocalization of new origins and sister replication forks did not occur, or was less extensive, in cells lacking SeqA. We also found that SeqA was capable of pairing sister oriC regions after DNA replication i*n vitro*. We suggested that colocalization of sister origins and replisome was independent of origin sequestration, but dependent on the presence of functional SeqA protein (Paper III). It might be that SeqA provide a coupling between initiation of replication, and origin and replisome organization during sequestration. After sequestration however, another mechanism might provide extensive pairing of these components.

Key experimental methods

Drug treatment of cells and flow cytometry analysis

Rifampicin is an antibiotic that inhibits the β-subunit of RNA polymerase (Messer, 1972;Lark, 1972;Zyskind *et al.*, 1977). Initiation of replication, but not elongation, depends on transcription by the RNA polymerase. Thus, treatment with rifampicin allows ongoing rounds of replication to finish and no new initiations of replication take place. Cephalexin is another antibiotic that inhibits cell septum formation and therefore cell division. Cells that are simultaneously treated with rifampicin and cephalexin will end up with an integral number of chromosomes that reflect the number of origins at the time of drug treatment. In a culture with cells that initiate replication synchronously will therefore ended up with 2^n (n = 1, 2, 3…) chromosomes, i.e., 2, 4 or 8 chromosomes, while cells that initiate asynchronously will also contain 3, 5, 6 or 7 chromosomes (Skarstad *et al.*, 1986).

Flow cytometry is a sensitive method for measuring the characteristics of individual cells *in vivo* (Boye and Lobner-Olesen, 1991). The cells are fixed in ethanol and stained with dyes that bind specifically to DNA or protein (Hoechst 33258 and FITC, respectively). By measuring the fluorescence emitted from single cells passing an excitation light-focus in the flow cytometer, the DNA and protein content per cell is determined. The protein content is proportional to the cell mass and determination of protein content is an alternative to light scatter measurements (Wold *et al.*, 1994). The analysis of thousands of cells from an exponentially growing culture or a culture treated with antibiotics is a way of determining the average cell mass, cell mass at initiation of replication, DNA content per cell and number of origins per cell.

The initiation age and the B, C and D periods were calculated on the basis of the model proposed by (Cooper *et al.*, 1968) and later described (Skarstad *et al.*, 1985;Molina *et al.*, 2004). The average number of replication forks per cell was calculated from the generation time, C and D periods (Molina *et al.*, 2004).

Light microscopy

Visualization of the bacterial cell, chromosome and protein was performed with light microscopy. The bacterial cell outline was observed using phase contrast or DIC objectives. The chromosome was visualized by staining DNA with DAPI fluorescent dyes. The origin region, newly synthesized DNA and the SeqA protein were visualized with methods described below.

Immunofluorescence microscopy was used to detect the SeqA protein or newly synthesized DNA within a cell using specific antibody. Newly synthesized DNA was labeled by incorporation of 5-bromo-2-deoxyuridine (BrdU) (Molina *et al.*, 2004). BrdU is a synthetic nucleoside which is an analogue of thymidine and is incorporated into the newly synthesized DNA of replicating cells. The cells were then fixed in methanol, attached to a microscope slide and treated with lysozyme in order to permeabilize the cell membrane. BrdU labeled DNA was detected using primary mouse anti-BrdU antibody conjugated to a fluorescent dye (Alexa488) (Molina *et al.*, 2004). The SeqA protein was detected in a two step reaction. The cells were fixed in ethanol, attached to a microscope slide and treated with lysozyme. The detection of SeqA included incubation with a primary rabbit anti-SeqA antibody and a secondary anti-rabbit antibody conjugated to a fluorescent dye (Cy3) (Paper I).

Immunofluorescence microscopy was then performed using a Zeiss Axioplan2 phase-contrast/fluorescence microscope equipped with a 63× objective and a BP546/12 filter (Cy3) or BP450/490 filter (Alexa488). DNA was visualized with a BP365/12 filter. Pictures were taken using a MicroMax CCD camera (Princeton Instruments) that was connected to a computerized image analysis system (Axiovision2 Multichannel, Zeiss) (Paper I).

Visualization of the origin region of the chromosome was performed with live cell microscopy. An array containing 240 repeats of the *lac* operator sequence (21 bp) was placed in the *att*Tn7 (3.909Mbp) site near *oriC* (3.910Mbp) (Lau *et al.*, 2003). The cells were then transformed with a plasmid containing *lac* repressor fused to green fluorescent protein (GFP-LacI) (Gordon *et al.*, 1997). GFP is a protein, comprised of 238 amino acids (27 kDa), from the jellyfish *Aequorea victoria* that fluoresces green when exposed to blue light (Prendergast and Mann, 1978;Tsien, 1998). Expression of GFP-LacI was

induced by addition of arabinose and GFP-LacI bound specifically to the *lac* operator array near *oriC*. After 90 minutes the cells were then transferred into a 0.7 % agarose layer containing phosphate-buffered saline for microscopy. Fluorescent microscopy imaging was performed with an Olympus Fluoview BX61 Laser Scanning Microscope equipped with an UPlanApo 100x objective, and BA 505-525 filter. Images were captured, processed, and analyzed using Flouview 4.3, MetaMorph 6.1 and Adobe Photoshop Elements 4.0 software (Paper III).

Reconstituted DNA replication, restriction enzyme analysis and native gel electrophoresis

DNA replication can be reconstituted *in vitro* using supercoiled *oriC* plasmid and purified proteins (Kaguni and Kornberg, 1984). We performed reconstituted DNA replication using the following proteins: DnaA, DnaB helicase, DnaC, single stranded DNA binding protein (SSB), integration host factor (IHF), DnaG primase, DNA gyrase and polymerase III holoenzyme essentially as described in (Wold *et al.*, 1998). We included primase at 150 nM, which supported correctly coupled bidirectional DNA replication (Hiasa and Marians, 1994). The replication proteins were incubated at 30^{o}C with negatively supercoiled *oriC* plasmid and deoxynucleotides in a buffer containing ATP and magnesium. Replication was measured as the amount of radioactively labelled deoxynucleotides incorporated into synthesized DNA.

The replication reaction was further analyzed in an experimental setup that included restriction enzyme digestion and native gel electrophoresis. The DNA replication reactions were digested with *Eco*RI and *Pst*I, yielding two fragments: a 678 base pair *oriC* fragment and a 2959 base pair plasmid fragment. The restriction digestion products were resolved by native agarose (1.2%) gel electrophoresis.

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